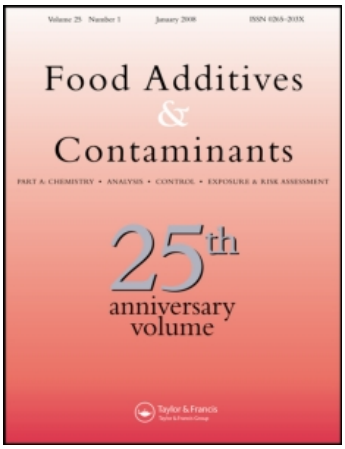

Food Additives & Contaminants: Part A
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713599661

Analysis of oxytetracycline residue in salmon muscle using a portable analyzer based on Eu^{III} luminescence

G. Chen [a]; G. Liu [b]
[a] US Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, Wyndmoor, PA 19038, USA [b] Shanghai Institute for Food and Drug Control, Shanghai 201203, China

First Published:August2009

To cite this Article Chen, G. and Liu, G.(2009)'Analysis of oxytetracycline residue in salmon muscle using a portable analyzer based on Eu^{III} luminescence',Food Additives & Contaminants: Part A,26:8,1172 — 1179
To link to this Article: DOI: 10.1080/02652030903013302
URL: http://dx.doi.org/10.1080/02652030903013302

Food Additives and Contaminants
Vol. 26, No. 8, August 2009, 1172–1179

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Analysis of oxytetracycline residue in salmon muscle using a portable analyzer based on Eu^{III} luminescence

G. Chen[a]* and G. Liu[b]

[a]*US Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, 600 E. Mermaid Lane, Wyndmoor, PA 19038, USA;* [b]*Shanghai Institute for Food and Drug Control, Shanghai 201203, China*

(*Received 31 December 2008; final version received 2 May 2009*)

In the United States, oxytetracycline (OTC) was approved by the Food and Drug Administration (FDA) for use in salmon, catfish and lobster; worldwide, it is the most prominent therapeutant in aquaculture. In this study, OTC and 4-epiOTC residues in salmon muscle were determined using a portable analyzer based on europium-sensitized luminescence (ESL). Extraction was performed first in EDTA/metaphosphoric acid/NaCl/hexane and then in metaphosphoric acid. After centrifugation, cleanup was carried out using hydrophilic–lipophilic balance cartridges. The ESL intensity, integrated over a 25–1000 μs interval, was linear ($r^2 = 0.9999$) over three orders of magnitude. In comparison to xenon flashlamp, light-emitting diode (LED) excitation reduced background noise by sixfold to 6.4 ng g^{-1}. Without prior chromatographic separation, this ESL method/instrument combination achieved 10.9 ng g^{-1} limit of detection and $<2\%$ average relative standard deviation. Average recovery was 82.0, 83.6 and 86.8% at 50, 100 and 2000 ng g^{-1}, respectively.

Keywords: extraction; screening assays; veterinary drug residues, tetracycline; fish

Introduction

World aquaculture has been growing at an 8.8% average annual rate since 1970. Salmon is among the most successful cultivated species: global farmed salmon production surpassed wild catch in 1998, and reached 1.3 million tonnes in 2005 (Liu and Sumaila 2008). Throughout cultivation, however, outbreaks of infectious diseases pose a serious threat. Due to broad-spectrum efficacy and cost advantage, oxytetracycline (OTC) is the most prominent therapeutant worldwide in treatment of a wide range of bacterial infections, including coldwater disease, columnaris, enteric red-mouth, fin rot, furunculosis, gill diseases and vibriosis. In the US, OTC was approved by the Food and Drug Administration (FDA) to use in salmon, catfish, and lobster. Consequently, OTC residue may be present in salmon muscle contributing to the emergence of antibiotic-resistant pathogens (Namdari et al. 1998; Aoyama et al. 1991). To protect public health, the tolerances of total tetracyclines (TCs) in edible animal muscle were set at 2 μg g^{-1} by the FDA, and the maximum residue limit (MRL) was set at 100 ng g^{-1} by the European Union (EU) as the sum of individual parent compound and its 4-epimer metabolite rather than total TCs. In a global economy, sensitive methods for determining OTC residue in this significant species are needed by farmers, traders, regulators, as well as researchers in pharmacokinetic and depletion studies.

Screening of OTC residue in fish tissue can be performed by microbiological inhibition assay, a nonspecific, insensitive and time-consuming process (Stehly et al. 1999). Microbial receptor assay (Charm II) and enzyme link immunosorbent assay (ELISA) improve sample throughput but fail to detect metabolites (4-epimers) (Munstedt et al. 2005), and test kits have limited shelf-life even at low temperatures. Binary screening results fulfill certain tasks; however, quantitative data are indispensable under many circumstances. To date, OTC determination in fish muscle is carried out by high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection (Coyne et al. 2004; Carignan et al. 1993; Bjorklund 1988), or fluorescence detection after post-column derivatization by UV radiation (Agasoster and Rasmussen 1992), or chelation with Mg^{2+} (Pena et al. 2003; Rupp and Anderson 2005). HPLC detection techniques applied to TC residues in other matrices include mass spectrometry (MS) (Carson et al. 1998) and electrochemical (EC) (Loetanantawong et al. 2004) for OTC in shrimp, and chemiluminescence (CL) for TCs in honey (Wan et al. 2005), of which EC was only marginally sensitive for regulatory purpose. HPLC provides powerful

*Corresponding author. Email: guoying.chen@ars.usda.gov

ISSN 0265–203X print/ISSN 1464–5122 online

DOI: 10.1080/02652030903013302
http://www.informaworld.com

separation, but it can be relatively slow and expensive. In addition to HPLC, other methods to determine TC residues in fish include bioluminescence (BL) (Pellinen et al. 2002), flow-injection analysis (FIA) with CL detection (Xiong et al. 2006), and capillary electrophoresis (CE) with UV detection (Huang et al. 1997).

To improve sample throughput, a possible strategy is to eliminate chromatography by employing a specific detection technique. It is a challenging task due to the chemical complexity of animal tissue and stringent regulatory tolerances. Two potential techniques, derivative spectrometry (Salinas et al. 1989) and chemiluminometry (Halvatzis et al. 1993), were developed for analysis of TCs in urine, honey and pharmaceutical compounds; but they were not sensitive enough to detect residues in foods. Fortunately, europium-sensitized luminescence (ESL) (Hirschy et al. 1983) provides much promise for this task, including very high sensitivity and specificity (Wenzel et al. 1988). It was applied to analysis of TCs in serum (Arnaud and Georges 2001), chlortetracycline (CTC) in urine and serum (Yang et al. 1997), TC in milk (Gala et al. 1997), and OTC in catfish muscle (Chen et al. 2004). All these applications relied upon commercial fluorescence spectrophotometers equipped with a xenon flashlamp as excitation source.

Xenon flashlamps provide distinct advantages, including a wide spectral range (from UV to IR), high intensity, high stability and long lifetime. As a white-light source, a xenon flashlamp covers of a broad range of target analytes, but also introduces excess noise in the frequency domain. On the other hand, its relatively long post-flash residual emission, characterized by lamp extinction time ($\sim$80 μs) and ion extinction time ($\sim$400 μs), introduces noise in the time domain. Furthermore, xenon plasma is produced by high-current discharge from a capacitor, notoriously creating transient noise, known as electromagnetic interference (EMI), in high-gain detection circuitry. The combined noises from these sources deteriorate signal-to-noise ratio (S/N) and, hence, detrimentally affect analytical sensitivity. Fortunately, these noises can be significantly reduced by replacing the xenon flash lamp with a UV light emitting diode (LED), thanks to its narrow emission bandwidth, clean post-pulse extinction and high energy efficiency. The only trade-off is the limited range of target analytes an LED can excite within its bandwidth. Because TC drugs account for >50% of total antibiotic use in world agriculture, we developed a specific TC analyzer based on LED excitation at 380 nm. The background noise of this analyzer in water is more than an order of magnitude lower than that of state-of-the-art fluorescence spectrophotometers equipped with xenon flashlamps. As a result of reduced noise and improved signal reproducibility, detection sensitivity was significantly enhanced (Chen 2008).

Salmon is among the most significant aquatic species worldwide and the most popular seafood in Europe, Japan and America. However, attempts to apply our catfish protocol to salmon muscle failed due to two problems: high fat content and poor recovery. Fat as high as 11% is found in farmed salmon fillets; if not properly separated, it would coat sorbent surfaces, affecting both retention and recovery during solid phase extraction (SPE) cleanup, or even completely clog SPE columns. Poor recovery was most likely due to analyte entrapment inside dense protein precipitates. In this study, a new cleanup protocol was specifically designed to overcome these problems and ESL was measured using the above-described analyzer. In comparison to our catfish protocol (Chen et al. 2004), overall analytical performance was improved. Use of portable instrumentation also made this method one step closer to field application.

Materials and methods

Chemicals and standards

OTC hydrochloride, reagents and solvents were purchased from Sigma-Aldrich (Milwaukee, WI, USA), and 4-epioxytetracycline (4-epiOTC) was purchased from ACROS Organic (Fair Lawn, NJ, USA). The reagent solutions include: 4 g l^{-1} metaphosphoric acid in 10 : 90 (v/v) methanol/water, saturated Na_2EDTA, saturated NaCl, 250 g l^{-1} cetyltrimethylammonium chloride (CTACl), 1 mM $Eu(NO_3)_3$–1 mM Na_2EDTA, and a 0.125-M tris(hydroxylmethyl)aminomethane (Tris) buffer at pH 8.5. A Barnstead E-pure system (Dubuque, IA, USA) was used to prepare deionized water for these solutions. OTC and 4-epiOTC stock solutions were prepared monthly at 1.00 g l^{-1} in methanol and stored in amber bottles at 4°C; further dilutions were performed daily with deionized water. Oasis HLB cartridges (60 mg) were purchased from Waters (Milford, MA, USA).

Apparatus

The ESL-based portable TC analyzer (Figure 1) has been reported elsewhere in more detail (Chen 2008). A UV LED generated 15 μs pulses at 380 nm (15 nm half width) for selective OTC excitation. The beam, roughly collimated by a quartz lens, passed a glass UV bandpass filter and illuminated a 1.0 × 1.0 cm fluorescence quartz cuvette. The resulting luminescence beam, collected at 90° by a lens, passed an interference filter and a longpass glass filter, and was finally detected by a gated photomultiplier tube (PMT). Its output photocurrent was amplified and filtered to eliminate high-frequency noise, and finally digitized and integrated. To correct pulse-to-pulse fluctuation, the energy of individual LED pulses was monitored by a

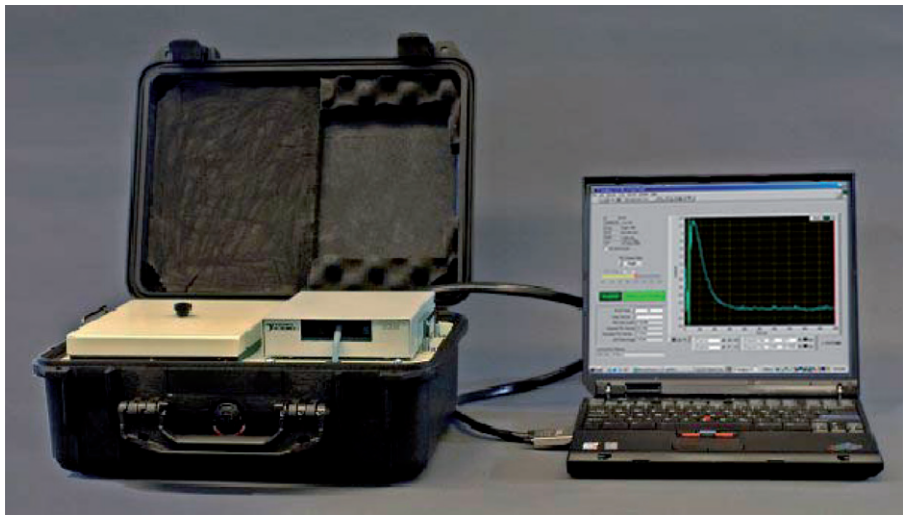

Figure 1. Portable analyzer displaying an ESL decay curve of 100 ng g^{-1} OTC in salmon muscle, along with measurement parameters and other results.

photodiode (PD) and used for signal normalization. Instrument operation and data processing were both controlled by a laptop computer running a custom LabVIEW program. Built in a water-tight polypropylene carrying case, this 7.4-kg analyzer was designed for field use, but can be used equally well in laboratories.

Extraction and clean-up

Salmon fillets, purchased from local food stores, were skinned, homogenized with a food processor and stored at −80°C. Portions of thawed muscle, 2.00 ± 0.03 g each, were placed in 50-ml polystyrene centrifuge tubes. The samples were spiked to desired levels with ≤200 µl of OTC or 4-epiOTC standard solutions of appropriate concentrations. The tubes were kept in the dark for 20 min with good muscle–solution contact. Next, 8 ml of 4 g l^{-1} metaphosphoric acid in methanol/water (10 : 90, v/v), 1 ml of saturated Na_2EDTA solution, 1 ml of saturated NaCl solution and 2 ml of hexane were added. The tubes were vortexed for 20 s and centrifuged at 4150 rpm for 10 min at room temperature. Four layers formed from bottom up: a solid pellet, an aqueous layer, a ~2-mm thick soft fat disc and an organic liquid layer. After removal of the top organic layer with a pipette, the aqueous phase was decanted slowly to a 50-ml centrifuge tube, allowing the soft fat disc to adhere to the wall. Next, the fat disk was removed with a spatula and 8 ml of 4 g l^{-1} metaphosphoric acid in methanol/water (10 : 90, v/v) was added to the pellet, followed by a 10-s vigorous hand shaking to loosen the pellet, a 20-s vortex mixing, and a 10-min centrifugation as before. The supernatants were combined, to which 1 ml of hexane was added, followed by a 20-s vortex mixing and another 10 min centrifugation step. The top organic layer was removed with a pipette. Next, Oasis HLB cartridges were installed on a vacuum manifold and conditioned stepwise with 2 ml of methanol and 2 ml of deionized water. The supernatants were then loaded slowly, followed by washing with 2 ml of deionized water. After the sorbent beds were dried under vacuum for 10 min, OTC was finally eluted with 2 ml of methanol to test tubes. During sample preparation, subdued lighting is recommended to minimize analyte photodegradation.

ESL measurement

To each eluate, the following reagents were added: 0.5 ml of 1 mM $Eu(NO_3)_3$–1 mM Na_2EDTA, 0.2 ml of 250 g l^{-1} CTACl and 3.3 ml of 0.125 M Tris buffer at pH 8.5. The test tubes were then vortexed for 5 s. Measurements were made in triplicate, each on a fresh ~2-ml aliquot. The early portion (0–25 µs) of the digitized signal was discarded; integration over a 25–1000 µs interval yields I_{ESL}. The normalized ESL signal, I_{ESL}/E_{pulse} where E_{pulse} was the energy of an individual LED pulse, was recorded as the final result. In each measurement, data were averaged over 20 pulse cycles which are user-definable.

Results and discussion

Sample preparation

Farmed salmon muscle contains ~20% protein, up to 11% fat and other minor components including minerals and vitamins (US Department of Agriculture 2008). Effective extraction media must overcome

OTC's propensity to bind to proteins and chelate multivalent cations (Anderson et al. 2005). Acids (HCl, H_3PO_4, trichloroacetic acid, metaphosphoric acid, succinic acid, etc.) or acidic buffers (glycine, phosphate, succinate, oxalic, McIlvaine, etc.) are used to denature proteins, sometimes with the aid of miscible organic solvents, such as alcohols or acetonitrile. EDTA, on the other hand, is effective to free TCs from their complexes with mineral ions. Mild acidic pH is necessary where the zwitterionic state dominates which is favored for sorbent adsorption during SPE cleanup. Among documented TC extraction media, EDTA–McIlvaine buffer at pH ~4 is the most prevalent for animal tissue matrices. When applied to salmon muscle, unfortunately, recovery fluctuated considerably, not only between samples of different origins but also between samples from the same fillet. These observations suggested a likely cause: analyte entrapment by relatively dense protein precipitate that formed after addition of extraction medium. It was found that by using $4\,g\,l^{-1}$ metaphosphoric acid in methanol (10 : 90, v/v), dense protein precipitates did not form. The pH of this medium (~3), as discussed above, was also ideal for OTC adsorption on SPE sorbent, which was further improved by addition of NaCl that enhanced ionic strength. Overall, this extraction protocol resulted in higher and more consistent recovery, as demonstrated by the experimental data in Table 1.

Salmon muscle has a much higher fat content than other fish species. If not removed, fat will coat SPE sorbent surfaces affecting flow pattern, retention, and recovery, or even completely clog the sorbent bed. Mechanically, fat could be successfully removed using a spatula when it formed a disc during centrifugation (Rupp and Anderson 2005). However, at lower fat contents, a complete fat disc does not form; whereas at higher fat contents, a fat disc is accompanied by a large number of tiny fat droplets. Unfortunately, it is very difficult to separate these droplets due to their small dimensions and large numbers. In contrast, defatting by liquid–liquid phase separation was more reliable using hexane (Agasoster and Rasmussen 1992; Pena et al. 2003) or dichloromethane. Due to health and environmental concerns, hexane was used in this work. After initial centrifugation, fat formed a ~2-mm translucent soft disc that adheres easily to the wall. To obtain clear supernatants, defatting was performed twice.

For analyte cleanup, liquid–liquid extraction (LLE) proved adequate for certain tissue matrices, wherein ethyl acetate performed better than other solvents (Poiger and Schlatter 1976). However, OTC is highly polar and, hence, soluble in water; so extra measures were needed to achieve reasonable recovery: a suitable pH to keep OTC in a zwitterionic state, ion pairing, and an aqueous phase saturated with salt. To switch back to an aqueous phase, ethyl acetate must be removed by evaporation, during which, unfortunately, analyte loss occurred (Rupp and Anderson 2005). With all these precautions, OTC still had the lowest recovery among TC members. To date, SPE remains the most prevalent sample preparation approach for TCs that fulfills cleanup, enrichment and phase change. With multiple functionalities available for adsorption, many sorbent types prove adequate for TCs cleanup. Oasis HLB was chosen in this work based on the following considerations: its poly(divinylbenzene-co-*N*-vinylpyrrolidone) macroporous backbone eliminates interactions between silica and polar OTC, and its polar functionalities improve both retention and wettability in aqueous media. As described below, this extraction-cleanup protocol consistently resulted in >80% recovery in the 50–2000 $ng\,g^{-1}$ range among samples of different origins.

Luminescence measurements

Most quantitative methods for OTC in salmon analysis rely upon powerful HPLC separation. Additional mass resolution is available if mass spectrometry (MS) is

Table 1. Background and recovery.

Samples	Background $0\,ng\,g^{-1}$	Recovery (%)		
		$50\,ng\,g^{-1}$	$100\,ng\,g^{-1}$	$2000\,ng\,g^{-1}$
Wild 1	7.1	78.96	81.15	87.38
Wild 2	5.9	87.75	83.26	88.72
Farmed 1	9.7	83.99	85.51	81.84
Farmed 2 (color enhanced)	5.0	76.16	79.34	91.91
Farmed 3 (organic)	5.3	83.09	85.74	88.09
Farmed 4	5.8	80.76	84.48	86.82
Farmed 5	6.6	80.97	83.79	82.85
Farmed 6	5.9	84.03	85.62	
Average	6.4	82.0	83.6	86.8
SD	1.5	3.6	2.3	3.5
RSD (%)	23.4	4.3	2.8	4.0

elected for detection. Without prior separation, any detection technique must rely upon its intrinsic specificity to overcome interference from concomitant matrix species. LED-excitation ESL excels in this aspect. Firstly, the LED's 380-nm emission band overlaps well with OTC's absorption peak, providing selective excitation. Secondly, OTC chelates Eu^{3+} to form a 1 : 1 Eu-OTC complex; intrachelate proximity and energy matching (OTC's T_1 at 18,100 cm^{-1} versus Eu(III)'s $^5D_0$ at 17,260 cm^{-1}) make energy transfer efficient and irreversible. Thirdly, Eu(III)'s $^5D_0$ to $^7F_2$ emission at 617 nm can be selectively detected using a narrow-band interference filter. Finally, the long excited-state lifetime enables time-resolved measurement to reject short-life fluorescence from interfering concomitant components. From an engineering point of view, performance of time-resolved measurement is further enhanced by a PMT-gating technique implemented in this analyzer; consequently, integration can start as early as 25 μs, improving S/N and overall sensitivity. With LED excitation, our intended objectives were fully achieved. When data were compared with those from a Varian fluorescence spectrophotometer (Cary Eclipse) equipped with a xenon flash lamp, background noise was reduced to 1/6 in muscle matrix and more than an order of magnitude in reagent water. With PMT as a photodetector, collection efficiency depends on its operation voltage: the higher the voltage, the higher the collection efficiency. Therefore, a higher operation voltage is preferred in actual measurement, as long as the photocurrent is within scale.

To take full advantage of the above mechanisms, several conditions must be met: a suitable pH, a micellar environment provided by a surfactant (CTACl) and a synergistic agent (EDTA) to exclude water molecules from Eu(III)'s coordination sites and prevent quenching. Because OTC is liable to photodegradation (Scales and Assinder 1973), fresh aliquots must be used for each measurement and the number of pulse cycles per measurement must be limited. Finally, due to common molecular structure and, thus, chemical and spectroscopic behavior, this method cannot distinguish members of the TC family, and metabolites and isomers from parent drugs. Further discussion will follow below.

Analysis of OTC and 4-epiOTC in salmon muscle

Eight salmon samples were analyzed in this study to evaluate sample-to-sample reproducibility, among which two were wild and the rest were farmed. They also varied in country of origin, species, color and fat content. Among the farmed samples, one was labeled organic and one color enhanced. As shown in Table 1, average background noise of blank samples corresponded to 6.4 ng g^{-1}, resulting in a 10.9 ng g^{-1} LOD at $S/N = 3$. The average background of wild salmon was almost identical to that of farmed salmon indicating negligible OTC levels in all samples. In comparison to the background noise of reagent water (Chen 2008), it was obvious that this background originated mainly from co-extracted matrix components. Without prior chromatography, this background was nevertheless satisfactory for regulatory purposes (2 μg g^{-1} tolerance in the US and 100 ng g^{-1} in EU). At 2 μg g^{-1}, the signal varied by 7.4% RSD. Average recovery was 82.0% (4.3% RSD, $n = 8$) at 50 ng g^{-1}, 83.6% (2.8% RSD, $n = 8$) at 100 ng g^{-1}, and 86.8% (4.0% RSD, $n = 7$) at 2 μg g^{-1}, respectively (Table 1). If the second extraction was skipped, recovery dropped to ~70%.

The results from a randomly selected farmed salmon sample without any special label, spiked at 2–10,000 ng g^{-1}, are shown in Figure 2a–d. Linear response spanned over three orders of magnitude ($r^2 = 0.9999$). Due to filter effects (Parker and Rees 1962), the slopes became progressively lower from Figure 2a to c; beyond 25,000 ng g^{-1}, data started to deviate from linear response (Figure 2d). The background signal in Figure 2a corresponded to 5.3 ng g^{-1}, and the LOD from this data set was 6.2 ng g^{-1}. The reported 10.9 ng g^{-1} LOD was comparable to those of HPLC–MS due to good data reproducibility: 1.7% average relative standard deviation (RSD) over all the data points. Figure 2a–d are replotted in log–log scale as a single chart (Figure 3), where excellent linear relationship remained with a 0.94 slope. The discrepancy of this slope from unity indicated presence of filter effects, which were also evident from the two upper data points. Deviation of the three lower data points from the straight line, on the other hand, was caused by increasing contribution from background noise.

In comparison to HPLC–MS^n technique (Cherlet et al. 2003), this method achieves comparable sensitivity at a fraction of instrument investment and assay cost. By eliminating HPLC, overall sample throughput was improved. The cost, however, was the loss of prior-detection separation capability and, hence, reduced identification power. Although ESL is highly specific to TC-class antibiotics as a whole, it cannot distinguish individual members, 4-epimers metabolites from their parents, or an enol tautomer from its keto counterpart. This is both an advantage and a limitation. From a pharmacokinetic point of view, entity identification is essential. From a regulatory point of view, information on individual identity is unnecessary because the legislation stipulates that the reported value should comprise the sum of an individual TC member and its metabolite. Furthermore, the equilibria between parents and metabolites and between tautomers can be slow and elusive depending on multiple

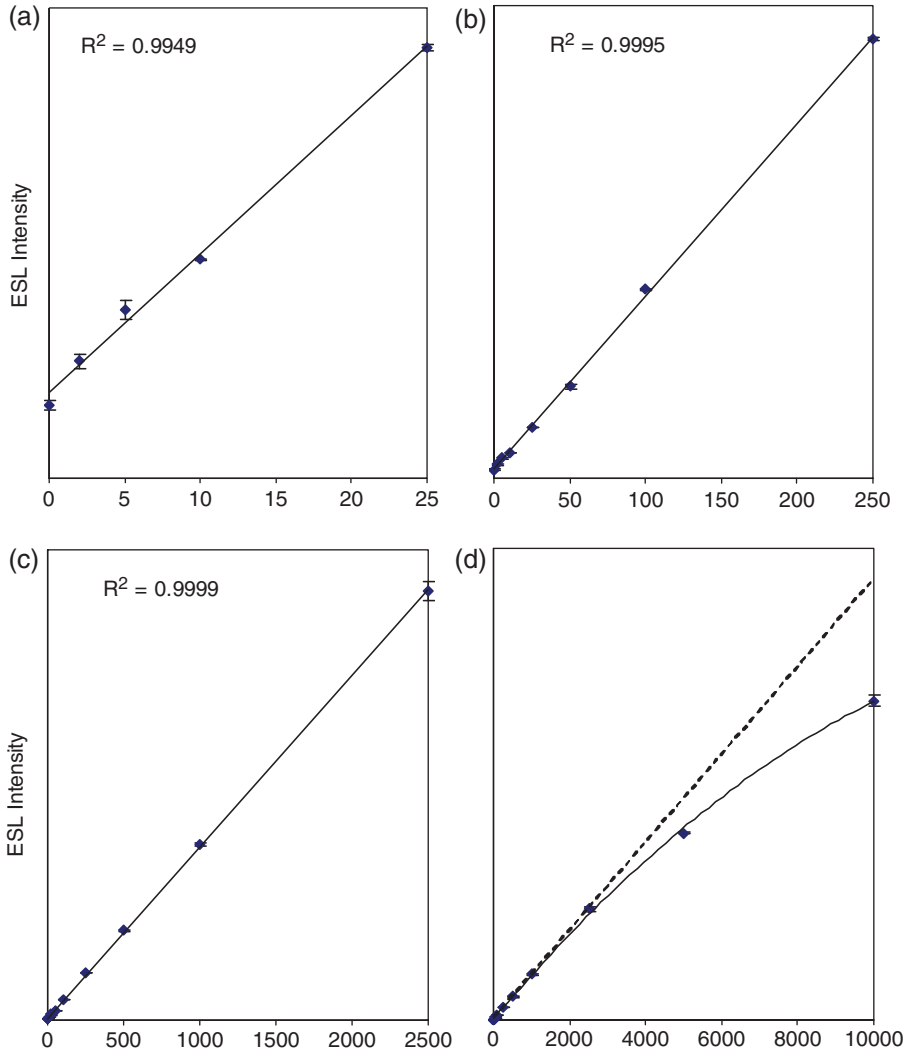

Figure 2. Calibration curves of OTC in salmon muscle in the 2–25 (a), 2–250 (b), 2–2,500 ng g^{-1} (c) and 2–10,000 ng g^{-1} (d) ranges. The error bars indicate ±1 standard deviation (SD).

factors (Blanchflower et al. 1997). Though it is possible to separate all the possible entities by HPLC–MS^n, then identify, quantify, and sum them together to report a single residue datum, this task is nevertheless complicated, demanding and expensive. In comparison, this ESL method bypasses all these complications. Simplicity in instrument operation and data interpretation enables personnel with less training to carry out depletion studies and residue monitoring.

Thus far, OTC is the only member in the TC class approved by FDA for salmon farming, and the most prominent antibiotic in aquaculture worldwide; however, if other TC drugs may be involved, identification is recommended prior to determination due to their varied luminescence responses (Gala et al. 1997; Arnaud and Georges 2001). To be valid and useful in residue analysis, this protocol–instrumentation combination must apply equally well to OTC and its metabolite, 4-epiOTC; so, a more relevant concern is whether these two compounds respond similarly. From the structural standpoint, they have identical molecular weights and elemental compositions but differ merely in the orientations of the $-N(CH_3)_2$ function at ring A (Figure 4). A series of experiments was performed to compare their behavior (Table 2). The ESL intensities in reagent water indicated their similar spectroscopic responses; whereas the ESL intensities and recoveries from spiked muscle matrix indicated their similar behavior in the overall extraction and cleanup processes. Based on these data, it is safe to claim equal applicability of this method to OTC and its 4-epimer metabolite, and the results are indeed indicative of the sum of both entities required by residue legislation. This is a definite advantage over microbial receptor assay (Charm II) and enzyme link immunosorbent assay that lack this ability.

Though this study was carried out in the laboratory, it is a step towards *in-situ* quantitative analysis in

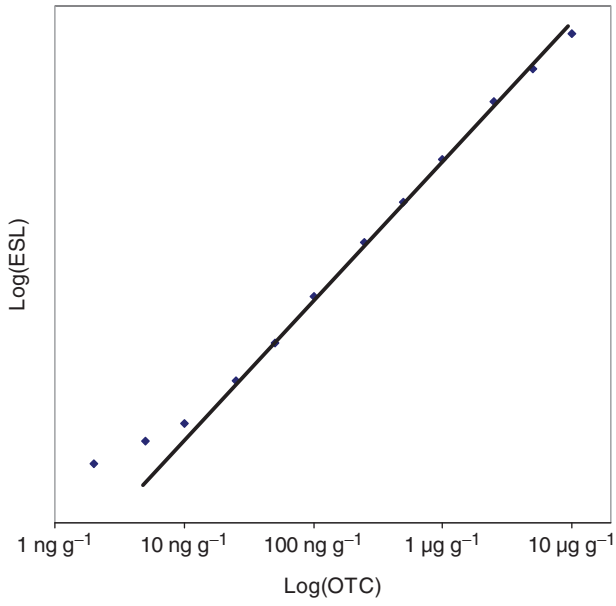

Figure 3. Log–log scale calibration curve of OTC in salmon muscle in the 2–10,000 ng g^{-1} range. The slope of the added line is 0.94.

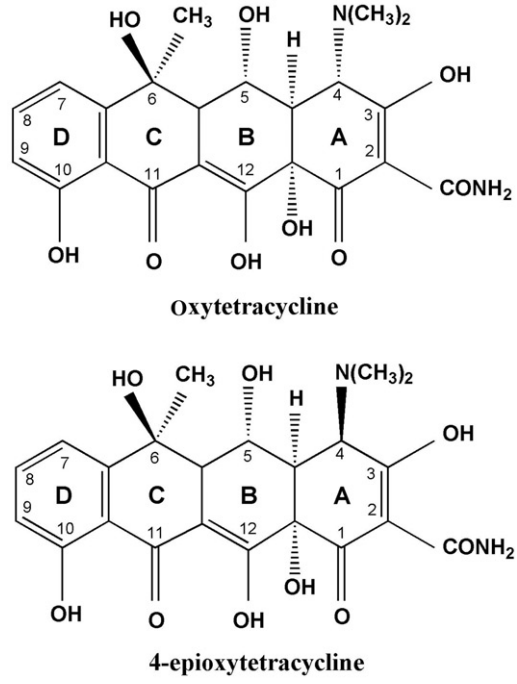

Figure 4. Molecular structures of OTC and 4-epiOTC.

Table 2. Relative ESL intensity and recovery.

	OTC	4-epiOTC
100 ng g^{-1} in water	100.0%	105.8%
100 ng g^{-1} in salmon muscle	100.0%	101.2%
Recovery at 100 ng g^{-1}	83.6%	82.9%

production sites or in the environment. This analyzer is light-weight and rugged for field deployment. Portable centrifuges are commercially available and the vacuum line can be replaced by a light-weight portable vacuum pump or eliminated with simple gravity flow. Potential benefits of field assay include fast results, cost saving, and timely decision making.

Disclaimer

Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

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